

## MODIFICATION OF HOST RESPONSES TO BACTERIAL ENDOTOXINS\*

### I. SPECIFICITY OF PYROGENIC TOLERANCE AND THE ROLE OF HYPERSENSITIVITY IN PYROGENICITY, LETHALITY, AND SKIN REACTIVITY

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The mechanism of tolerance to the pyrogenic activity of Gram-negative bacterial endotoxins is most often attributed to a non-specific increase in the activity of the reticuloendothelial system (RES) (1). Recently, it was shown that Group A streptococcal exotoxins produced biphasic fever responses in rabbits (2). In contrast to the non-specific tolerance induced by endotoxins, three distinct toxins were identified based on their ability to induce specific pyrogenic tolerance; in addition, the pyrogenic activity was neutralized specifically with antiserum.

These observations suggested that, in addition to the non-specific RES activity, specific immune mechanisms may contribute to pyrogenic tolerance to Gram-negative bacterial endotoxins. Because endotoxins from organisms of different species and families induce non-specific pyrogenic tolerance, it is assumed that specific immunological mechanisms are not involved. If, however, there exist unsuspected common or cross-reactive antigens contributing to the pyrogenic activity, the non-specific nature of the mechanism would be more apparent than real. Our approach involved, therefore, an attempt to demonstrate specificity by the use of cross-tolerance tests as applied to the streptococcal pyrogenic toxins (2). Purified endotoxins were selected for this purpose on the basis of suspected chemical differences.

In addition, there is evidence to implicate the immunological state of the host in many of the biological activities of endotoxins. From birth, animals are continually exposed to Gram-negative bacterial endotoxins derived from organisms growing in the gastrointestinal tract. Of particular interest is the enhanced susceptibility of animals to endotoxins after colonization with Gram-negative bacteria (3); here there was evidence of specificity because the induced susceptibility was greater when the endotoxin was derived from the

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sensitizing strain. Too, the great resistance of young animals to the lethal effect of endotoxin with the gradual increase in susceptibility with age is compatible with the concept that an acquired delayed hypersensitivity plays a role in the modified response (4, 5).

This paper presents the results from two related studies; one demonstrates specificity in pyrogenic tolerance by the use of selected endotoxins and their derivatives; the other concerns the role of hypersensitivity in various biological activities of endotoxin including pyrogenicity, lethality, and skin reactivity. These, with additional observations, favor an immunological interpretation of pyrogenic tolerance and other host responses to bacterial endotoxins.

### *Materials and Methods*

*Animals.*—Male and female American Dutch rabbits were used. They were supplied from a single source and maintained on a diet of Nutrena pellets. Animals were quartered in air-conditioned rooms and all experiments were performed in an adjoining laboratory at the same temperature.

*Toxins.*—The endotoxins were generously donated by Dr. Westphal and Dr. Lüderitz of the Max Planck Institute for Immunobiology, Freiburg, West Germany. The following toxins were used and the designations in parentheses will be used throughout this paper: *Escherichia coli* 08-COO8<sub>2158</sub>S<sub>2</sub> (COO8); *Escherichia coli* 08-Lipid A<sub>11</sub>M<sub>1</sub> (Lipid A); *Salmonella abortus equi*-AE<sub>1850</sub>S<sub>2</sub> (AE); *Chromobacterium violaceum* NCTC 9694 (CV). *Salmonella typhosa* 0901 (ST0901) was obtained from Difco Laboratories, Inc., Detroit and Group A *Streptococcus pyogenes* type 18-C (T-18) was prepared in our laboratory (2).

*Preparation of stock solutions.*—(a) *Lipopolysaccharide endotoxins (LPS)*. 10 mg of purified LPS were dissolved in 1 ml of pyrogen-free sterile distilled water and heated in a boiling water bath for 2 minutes; this was then diluted with 9 ml of pyrogen-free sterile sodium phosphate-buffered saline, pH 7.0, 0.15 M to give 1 mg/ml. These solutions were stored at -20°C. (b) *COO8 Lipid A<sub>11</sub>M<sub>1</sub> (Lipid A)*. 10 mg were dissolved in 1 ml of pyridine and centrifuged. The small amount of residue was washed with an additional 0.5 ml of pyridine. The Lipid A-pyridine solution was added dropwise to 10 ml of pyrogen-free distilled water while stirring. A nearly clear, stable suspension was obtained. Merthiolate was added to give a concentration of 1/10,000. Pyridine was removed under vacuum at 30°C until the solution was reduced to a volume of 7 ml; an additional 5 ml of water was added and again reduced to 7 ml. Finally the solution was brought to a volume of 10 ml with distilled water to give a concentration of 1 mg/ml. This stock solution remained stable and active at 4°C for several weeks. It is important not to freeze Lipid A preparations because the suspension will be broken and activity reduced. Pyridine, even if not removed, was not pyrogenic or toxic in rabbits at concentrations present in working solutions.

*Working Solutions.*—Stock solutions were diluted with pyrogen-free sterile buffered saline to make the desired concentrations just before each experiment; these solutions were maintained at 3-4°C.

*Reagents.*—Low molecular weight dextran, merthiolate and all diluents were not pyrogenic when tested in rabbits.

*Determination of Febrile Response.*—All animals were conditioned 4 to 6 hours, 1 day before use. For temperature determinations, an animal rack with a capacity for 15 rabbits designed by the Chemical and Pharmaceutical Industry Company, Inc., New York, was used in conjunction with the electric universal thermometer, TE-3. Thermocouples were inserted 2 to 3 inches into the rectum and remained there throughout the experiment. Animals were conditioned for at least 1 hour before the injection of toxin to establish the baseline

temperature of individual rabbits; those with a temperature greater than 103°F were not used. Temperatures were recorded at 30 minute intervals for a period of 5 hours or longer. Many control runs were made using this equipment to determine the normal variation under the conditions of the experiments. A rise of 1°F is considered a significant response. The large variation in febrile response between individual animals makes it necessary to use at least 5 animals for each preparation.

*Minimal Pyrogenic Dose-3.*—Pyrogenic tolerance described by Beeson (1), concerns primarily the 3 hour portion of the fever curve, and it soon became apparent that in studies on tolerance, the minimal pyrogenic dose (MPD) (6), the fever index (FI) (1) and the newer fever index (FI<sub>40</sub>) (7) are not the most satisfactory units for quantitating the pyrogenic dose when measuring tolerance. This was quite apparent to Moses and Atkins (8) when working with EP tolerance; there as here, the changes in the fever contours were more important than fever indices in establishing the tolerant state. In some cases, the areas under the curves remain unchanged despite real alterations in the reactivity of the host; this is especially evident in the tolerance produced by Noll and Braude (9) with chemically modified endotoxins. Where the standard MPD unit is used, the slope of the dose-response curves for two different toxins may be markedly different but the MPD will be the same; this is illustrated by Neter *et al.* (10) where an endotoxin was treated with periodate and compared with the unmodified toxin. For these reasons, a new unit, MPD-3 (minimal pyrogenic dose-3 hours) is used in this investigation. It is defined as the smallest amount of toxin giving a mean rise of 1°F, 3 hours after intravenous injection. Each toxin is tested at 3 to 6 concentrations and each concentration is tested in at least 5 adult rabbits (1.0 to 1.2 kg). The mean febrile response at 3 hours is plotted against the log of the concentration. The best line is drawn to 1°F and the quantity of toxin at the intercept represents the MPD-3.

For each endotoxin there is a range of concentrations where a linear dose response can be obtained. If the concentration of toxin is too great, shock will be manifested in the animal by a rapid fall in temperature. A dose of 100 MPD-3/kg falls within the upper portion of the linear dose-response curve and therefore gives the maximal febrile response; this dose has been used in all of the cross-tolerance studies reported. To compensate for differences in the activity of different preparations, all toxins were titrated and adjusted to the desired MPD-3 level. By this method, the MPD-3 of these toxins in  $\mu\text{g}/\text{kg}$  were as follows: COO8, 0.008; AE, 0.004; CV, 0.003; Lipid A, 0.1; ST0901, 0.01; and T-18, 4.0.

*Pyrogenic Tolerance.*—The following injection schedule was used to develop pyrogenic tolerance. After the control test with 100 MPD-3/kg on day 1, daily stepwise increasing doses (MPD-3/kg) were injected intravenously into adult rabbits (1.0 to 1.2 kg) as follows: 200, 200, 400, 400, 600, 600; finally on day 8 or 9, pyrogenic tolerance to the homologous toxin was determined by testing with 100 MPD-3/kg. With Lipid A and CV toxins, maximal tolerance was induced when they were suspended in 0.5 per cent pyrogen-free dextran.

## RESULTS

### *A. Specificity of Pyrogenic Tolerance*

The following experiments represent reciprocal cross-tolerance tests with three pairs of purified endotoxins. The toxins in the first pair, isolated from *E. coli* 08 (COO8) and *S. abortus equi* (AE), are known to differ in their O specificity; those in the second, including *C. violaceum* (CV) and COO8, are thought to differ not only in O specificity but in some other portion of the macromolecule; the third pair include the parent COO8 toxin and its derivative Lipid A.

1. *AE and COO8.*—Two groups of rabbits were made tolerant to 100 MPD-

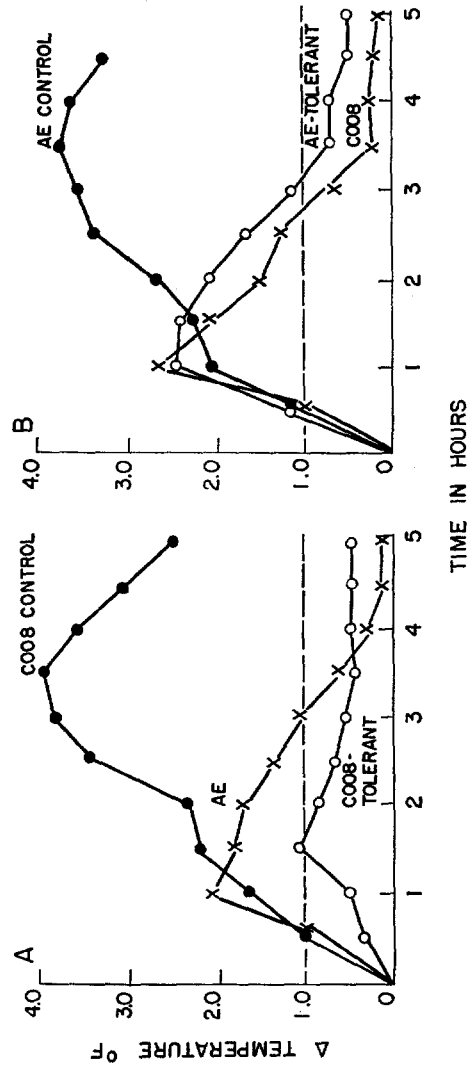


FIG. 1. Reciprocal cross-tolerance tests between COO8 and AE endotoxins. A, COO8-tolerant rabbits tested with heterologous AE endotoxin; B, AE-tolerant rabbits tested with heterologous COO8 endotoxin. Each curve represents the mean febrile response of 7 rabbits injected intravenously with 100 MPD-3/kg of endotoxin.

3/kg of the homologous toxin. On the following day, 100 MPD-3/kg of the heterologous toxins were injected. Results given in Fig. 1 show nearly complete reciprocal cross-tolerance. These results confirm those of Beeson (1), and serve as controls for the following experiments.

2. *CV and COO8*.—During our attempt to find endotoxins of different specificities, Dr. D. A. L. Davies, Microbiological Research Establishment, Porton, England, recommended to us the endotoxin of *C. violaceum* (CV). Crumpton and Davies (11) have reported the presence of D-fucosamine in the lipopolysaccharide isolated from this species but whether or not this explains the results of this experiment is not known. As in the preceding experiment, two groups of rabbits were made tolerant with the CV and COO8 endotoxins. As given in Fig. 2 A, COO8-tolerant animals showed little tolerance when given the heterologous CV toxin. In the reciprocal cross-tolerance tests, animals made tolerant to the CV toxin were equally tolerant to the heterologous COO8 toxin (Fig 2 B). These results, in contrast to the previous experiment, show a significant specificity in tolerance and are consistent with results one might expect with classical immune systems involving non-reciprocal cross-reacting antigens.

3. *Lipid A and COO8*.—Endotoxins prepared by the phenol method of Westphal *et al.* (12) contain mostly polysaccharide, lipid, and small quantities of peptide. Lipid A is obtained from the lipopolysaccharide by acid hydrolysis (13). Its chemistry is not completely known but its main components are D-glucosamine phosphoric acid ester, long chain fatty acids, and a considerable amount of  $\beta$ -hydroxymyristic acid; this derivative is about one-tenth as active biologically as the parent lipopolysaccharide.

When rabbits were made tolerant to Lipid A (Fig. 3 A) and given 100 MPD-3/kg of the parent COO8 toxin, the tolerance was slight. The same animals were not tolerant to exotoxin T-18 which confirmed our earlier results (2) on the failure of Gram-negative bacterial endotoxins to induce tolerance to the unrelated pyrogenic toxins of the Group A streptococci. Rabbits made tolerant to the parent COO8 toxin (Fig. 3 B) were partially tolerant to the derivative Lipid A. Again animals tolerant to the COO8 toxin were not tolerant to the streptococcal T-18 toxin.

4. *Anamnestic Response in Pyrogenic Tolerance*.—The short duration of tolerance has been used as evidence against an immunological mechanism (1). It should be noted, however, that in the induction of tolerance, animals are usually given the minimal number of injections to develop the tolerance; hyperimmunization in the usual sense is not attained. Also, the animals are tested by injecting the toxin intravenously and although animals may have a good recall mechanism, there is not sufficient time for it to respond. The results given in Fig. 4 show that on the 8th day after repeated daily injections, the animals were tolerant and, as previously shown by Beeson (1), they appeared to have lost tolerance on the 35th day. A single injection of 100 MPD-3/kg of the

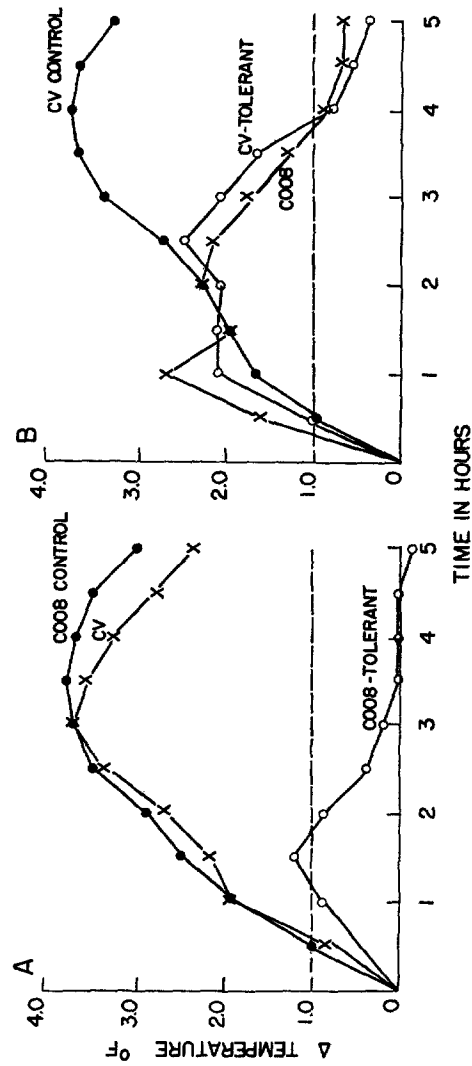


FIG. 2. Reciprocal cross-tolerance tests between COO8 and CV endotoxins. *A*, COO8-tolerant rabbits tested with the heterologous CV endotoxin. *B*, CV-tolerant rabbits tested with the heterologous COO8 endotoxin. Each curve represents the mean febrile response of 7 rabbits injected intravenously with 100 MPD-3/kg of endotoxin.

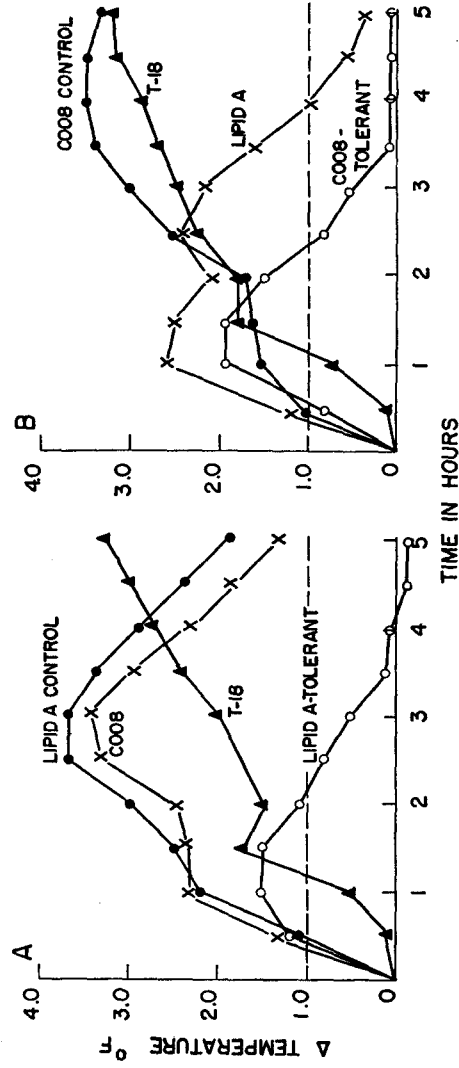


FIG. 3. Reciprocal cross-tolerance tests between COO8 and its derivative, Lipid A. *A*, Lipid A-tolerant rabbits tested with the parent COO8 endotoxin and T-18 exotoxin. *B*, COO8-tolerant rabbits tested with its derivative, Lipid A, and T-18 exotoxin. Each curve represents the mean febrile response of 8 rabbits injected intravenously with 100 MPD-3/kg of endotoxin or 10 MPD-3/kg of T-18 exotoxin.

homologous toxin was sufficient to give nearly complete tolerance within 2 days. The magnitude of this anamnestic response seems comparable to that observed with classical immune systems.

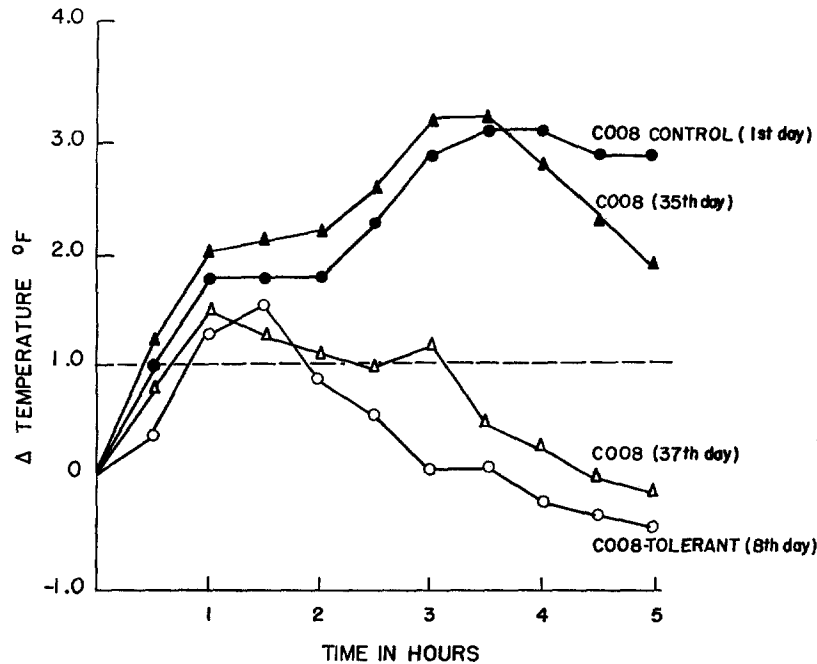


FIG. 4. Anamnestic response to endotoxin. Each curve represents the mean febrile response of 7 rabbits injected intravenously with 100 MPD-3/kg of COO8 endotoxin.

#### *B. Role of Hypersensitivity in Pyrogenicity, Lethality, and Skin Reactivity*

As stated previously, in view of the ubiquity of endotoxins in the gastrointestinal tract, any investigation of the mechanisms of pyrogenic tolerance and other biological activities of endotoxins should take into consideration the possibility that acquired delayed hypersensitivity might modify the host response. In the following experiments, various biological activities of endotoxins are tested in young and adult rabbits in an attempt to understand the mechanisms of acquired host responses to endotoxin.

1. *Effect of Age on Lethal Effects of Endotoxins in Rabbits.*—If young animals are resistant to endotoxins and become susceptible when adults as a result of the development of hypersensitivity to the endotoxin as postulated, it might be possible to prevent the enhanced susceptibility by inducing immunological paralysis in the sense of Felton (14). For this experiment, it was necessary to



determine the maximum amount of toxin that young animals could resist in a single injection. Also, the lethal effect of endotoxin was determined in rabbits of various ages.

The remarkable resistance of the young rabbit to endotoxin in contrast to

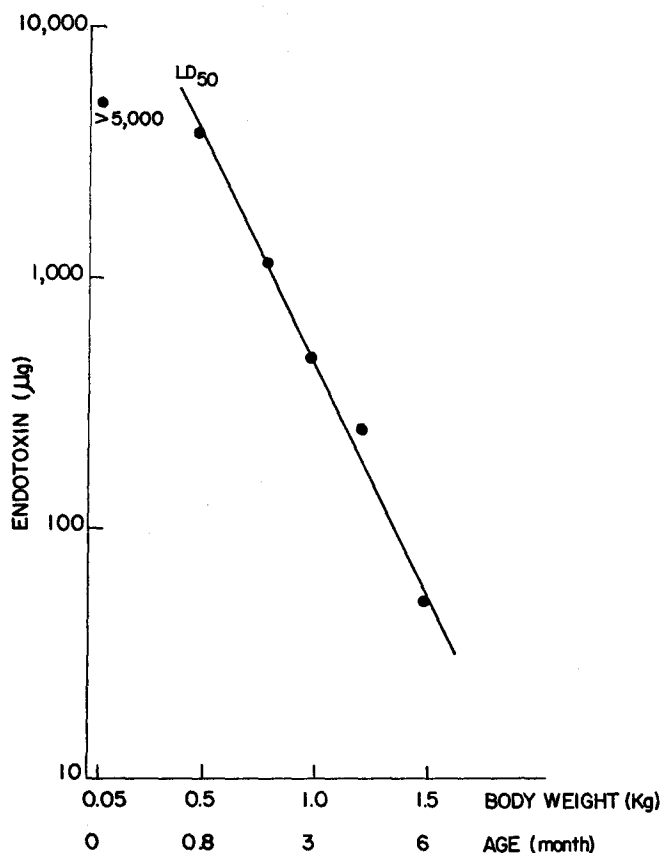


FIG. 5. Effect of age on lethality to endotoxin. 224 rabbits varying in age from newborn to over 6 months were injected with varying concentrations of ST0901 endotoxin intravenously. LD<sub>50</sub> calculated by the method of Reed and Muench (15).

the great susceptibility of the adult is shown in Fig. 5. Susceptibility increased linearly with age; the LD<sub>50</sub> in the resistant neonatal animal was greater than 5.0 mg/kg while in the susceptible adult the LD<sub>50</sub> was 50 µg/kg. These results are essentially the same as reported by Smith and Thomas (4) for rabbits, but more striking than those reported by Miler (5) for rats.

2. *Effect of Age on Pyrogenic Response to Endotoxins.*—If the 3 hour portion

of the fever curve is influenced by the degree of hypersensitivity of the host to some part of the endotoxin, as previously suggested, one would expect significant differences in the contours of the fever curves obtained in young and adult animals. Fig. 6 compares the mean fever responses of adult and young rabbits at two doses of endotoxin. Considerable differences in heights and contours of the fever curves, especially in the region of 3 hours were noted.

Our results do not confirm Smith and Thomas (4) who showed little differ-

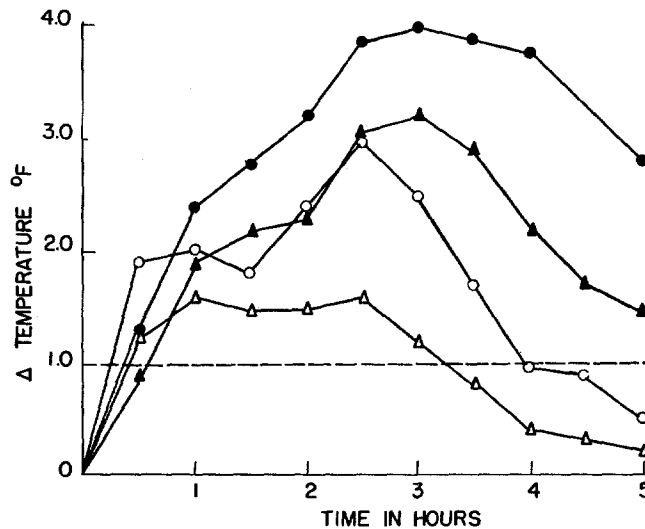


FIG. 6. Effect of age on pyrogenicity to endotoxin. *Adult*, each curve represents the mean febrile response of 10 rabbits, 1.0 to 1.2 kg at 3 months. *Young*, each curve represents the mean febrile response of 10 rabbits, 0.3 to 0.4 kg at 3 weeks. Doses of COO8 endotoxin are given. ●, adult 100 MPD-3/kg; ▲, adult 10 MPD-3/kg; ○, young 100 MPD-3/kg; △, young 10 MPD-3/kg.

ence in the pyrogenic response in young and adult rabbits. Some of the discrepancy could be accounted for by differences in dose. They gave the young (0.5 kg) and the adult (2.4 kg) the same dose of toxin; in our experiments, the dose was always adjusted to the weight of the animal. In addition, the difference in susceptibility to lethality between their young and old rabbits was considerably less than reported here, indicating that their young rabbits were older than ours, and, therefore, perhaps more sensitized. We have found a close correlation between the intensity of the febrile response at 3 hours and the lethal effect of endotoxin.

*3. Modification of Pyrogenic Response in Adult Rabbits by Their Early Exposure to Massive Doses of Endotoxin.*—As postulated previously, a massive dose of endotoxin given to the young animal might alter the development of hyper-

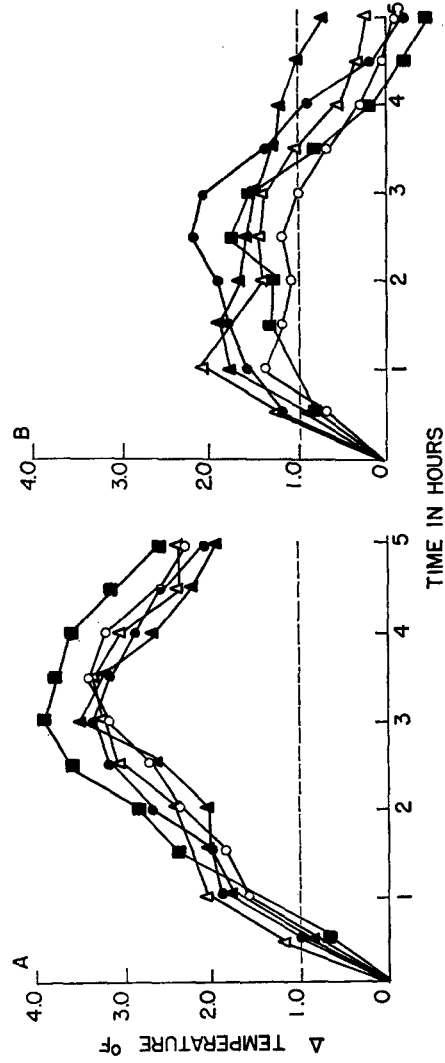


Fig. 7. Modification of pyrogenic response in adult rabbits by their early exposure to a massive dose of endotoxin. *A* (control), mean febrile response of normal adult rabbits (1.0 to 1.2 kg) to 100 MPD-3/kg of ST0901 endotoxin. Each curve represents the mean febrile response of 4 to 9 rabbits. *B* (test), litter mates of above controls randomly divided and injected with 4 mg/kg of ST0901 endotoxin (LD<sub>50</sub>) when they were young (3 weeks). Each curve represents the mean febrile response of 4 to 10 rabbits injected intravenously with 100 MPD-3/kg of ST0901 endotoxin when they became adult 2 months later (1.0 to 1.2 kg).

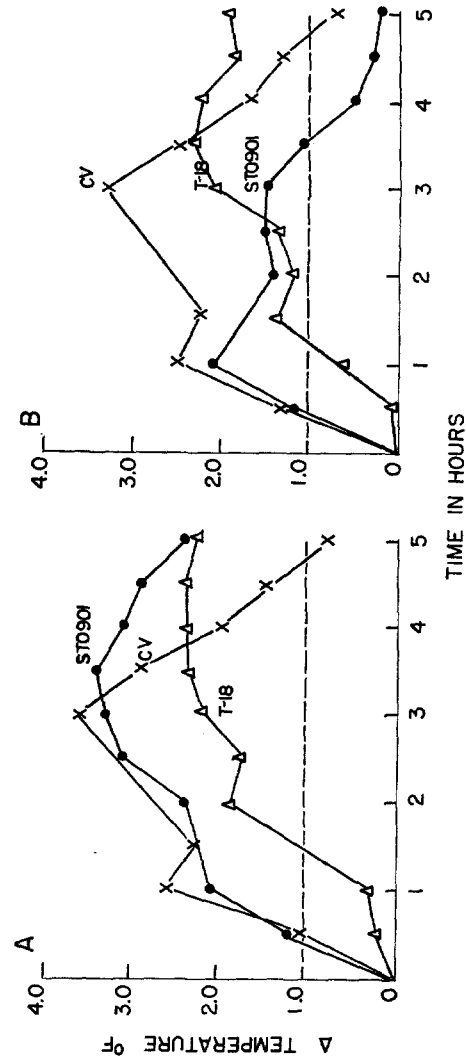


FIG. 8. Specificity of tolerance in adult rabbits given a massive dose of endotoxin when they were young. *A*, (control), each curve represents the mean febrile response of 9 adult rabbits (1.0 to 1.2 kg) injected intravenously with 100 MPD-3/kg of ST0901 and CV endotoxin and T-18 exotoxin. *B*, (test), each curve represents the mean febrile response of 6 litter mates (1.0 to 1.2 kg) of the above controls to 100 MPD-3/kg of homologous ST0901 and the heterologous CV and T-18; these animals received 4 mg/kg of ST0901 endotoxin when they were young (3 weeks).

sensitivity. Several litters of young rabbits (0.3 to 0.4 kg) were mixed and then randomly divided into two groups. Half of the animals were injected with a LD<sub>50</sub> of ST0901 endotoxin 4 mg/kg and the remainder served as controls. In each of the five experiments, approximately half of the injected animals survived; these, with litter mate controls, were raised under identical conditions until adult (1.0 to 1.2 kg) and all were injected with 100 MPD-3/kg of ST0901 endotoxin. Those adult animals given a massive dose of toxin when they were young (*B*), when compared with the controls (*A*), showed a marked suppression of the febrile response, especially at 3 hours (Fig. 7). Thus, a single massive dose of endotoxin, given to young rabbits at 3 weeks of age, can modify their febrile response when they become adults 2 months later.

Because a LD<sub>50</sub> dose was used to select the test animals at 3 weeks of age, the method is open to criticism. It might be assumed that the more resistant animals had been selected and might be expected to give a suppressed febrile response as adults. If this were so, one would expect that at least half of the litter mate controls corresponding to the half which survived the 4 mg/kg dose would also show the same suppressed febrile response when given the 100 MPD-3/kg as adults. To test this, we plotted the distribution of the febrile responses of individual rabbits taken at 3 hours after the injection of 100 MPD-3/kg both for the controls and for the tests. The median response for the controls was 3.5°F in contrast to 1.2°F for the test group. If selection had been a factor, the median of the lower half of the controls should have approached 1.2°F rather than the observed 3°F. Since there was no evidence of selection, we believe that the massive dose of toxin modified the febrile response by one of the two mechanisms suggested.

*4. Specificity of Pyrogenic Tolerance in Adult Rabbits Given an Early Exposure to a Massive Dose of Endotoxin.*—In an earlier experiment, purified endotoxin (CV) from *C. violaceum* gave a non-reciprocal cross-reaction when tested against endotoxin (COO8) from *E. coli*; with the pyrogenic exotoxin from Group A streptococcus type 18, there was complete specificity.

Adult animals, injected with massive doses of endotoxins when they were young, were tested with homologous ST0901 and the heterologous CV and T-18 toxins (Fig. 8 *B*); the controls were given the same toxins and represent litter mates of the test group (Fig. 8 *A*). Again, there was no difference in the 1 hour portion of the curve with the three toxins in either group. In the 3 hour portion of the curve (Fig. 8 *B*), however, there was a considerable suppression of the febrile response to the homologous ST0901, but with the heterologous CV and T-18 toxins, there were no significant differences between the control and test groups. With this method, therefore, we have shown the same degree of specificity observed when the toxins were tested in animals made tolerant by the method of Beeson (1).

*5. Effect of Age and Endotoxin Treatment on the Skin Reactivity to Endotoxin.*—

Regardless of the mechanism, if hypersensitivity is suppressed when young animals are given massive doses of endotoxin, it might also modify the host response to skin reactivity. Such a skin test has been used for quantitating

TABLE I  
*Effect of Age and Endotoxin Treatment on Skin Reactivity to Endotoxin*

Group	Weight <i>kg</i>	Age <i>months</i>	Endotoxin ST0901, $\mu\text{g}$ in 0.1 ml, intradermally*					
			100		10		1	
			Posi- tive/ total	Posi- tive	Posi- tive/ total	Posi- tive	Posi- tive/ total	Posi- tive
			<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
Endotoxin treated†	1.0 to 1.2	3	33/34	97	10/34	29	0/34	0
Litter mate control of the above group	1.0 to 1.2	3	30/30	100	30/30	100	11/30	37
Young rabbit control	0.3 to 0.4	0.8	4/8	50	0/8	0	0/8	0
Adult rabbit control	1.0 to 1.2	3	15/15	100	15/15	100	7/18	39

\* Positive skin reactions were greater than  $5 \times 5$  mm at 24 to 48 hours.

† Adult rabbits which had received ST0901 endotoxin 4 mg/kg when they were young (0.3 to 0.4 kg at 3 weeks).

TABLE II  
*Induced Resistance to the Lethal Effect of Endotoxin*

Experiment	Group	Body weight <i>kg</i>	Endotoxin ST0901 intravenously $\mu\text{g}/\text{kg}$	Results		
				No. of animals	No. of deaths	Mortality <i>per cent</i>
A*	Test	1.0 to 1.2	4000	10	0	0
	Control	1.0 to 1.2	4000	11	11	100
B	Test	1.0 to 1.2	2000	9	0	0
	Control	1.0	500	9	5	56
		1.2	250	11	5	45

\* A. Test, adult rabbits which had received 4.0 mg/kg of ST0901 endotoxin when they were young (0.3 kg). Control, normal adult litter mates of A (test).

B. Test, adult rabbits made tolerant by repeated daily injections of ST0901 endotoxin as follows: 1, 2, 2, 4, 4, 6, and 6  $\mu\text{g}/\text{kg}$ . Control, normal adult rabbits.

endotoxin toxicity (16) and the reaction has been compared with that seen in classical delayed-type hypersensitivity skin reactions (17).

As summarized in Table I, there is a considerable difference in skin reactivity between young and adult animals. Although this correlates well with the pyrogenic activity in these two groups, the failure of young rabbits to react to less

than 100  $\mu\text{g}$  may be due to lack of physiological maturation of the skin as suggested by Sterzl and Hrubesova (18). The fact remains, however, that a number of these young animals did react to 100  $\mu\text{g}$ .

We could also suppress the skin reactivity in adults by injecting massive doses of endotoxin (4 mg/kg) when the animals were young. To demonstrate this, it was necessary to titrate the toxins in the skin as shown in Table I. Significant differences were evident at 1 and 10  $\mu\text{g}$  levels, but not at the 100  $\mu\text{g}$  level. It is possible that the primary toxicity was contributing to the skin test directly and also augmenting the hypersensitivity. Such a mechanism has been suggested for the Dick reaction where the Group A streptococcal exotoxins appear to enhance a hypersensitivity reaction in the skin of older rabbits (2). The inhibition of the skin reaction correlates with the suppression of the 3 hour febrile response in similarly treated animals (Fig. 7).

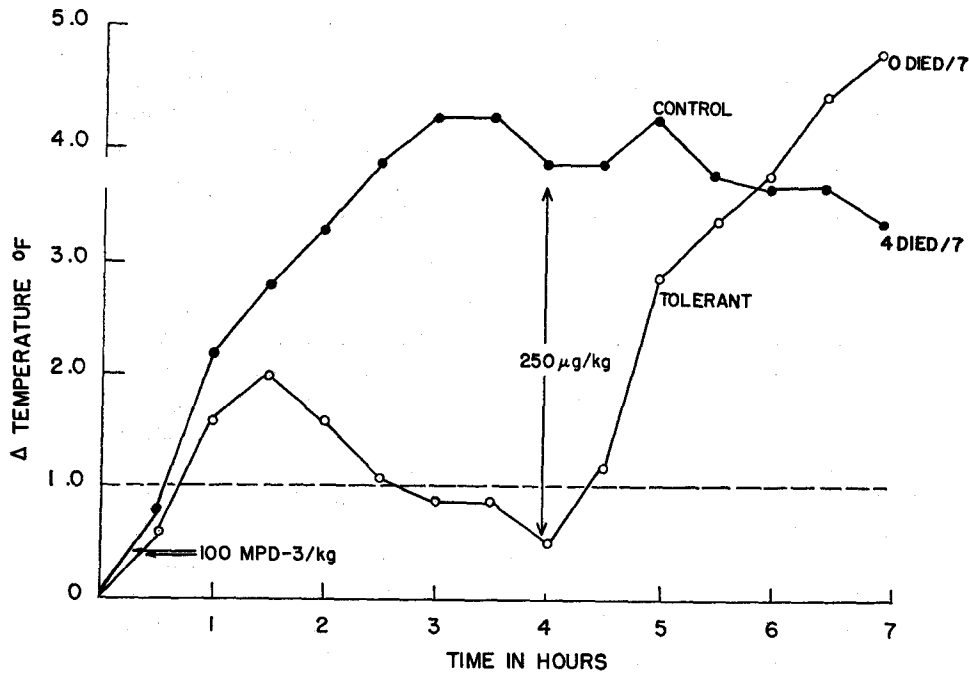


FIG. 9. Resistance of pyrogenic tolerant rabbits to endotoxin lethality. *Control*, mean febrile response of 7 normal adult rabbits (1.0 to 1.2 kg) given ST0901 endotoxin 250  $\mu\text{g}/\text{kg}$  4 hours after an initial injection of 100 MPD-3(1.0  $\mu\text{g}/\text{kg}$ ). *Tolerant*, mean febrile response of 7 tolerant rabbits (1.0 to 1.2 kg) given the same dose of ST0901 toxin as the controls.

6. *Induced Resistance to the Lethal Effect of Endotoxin.*—In addition to investigating the modification of the host response to pyrogenicity and skin reactivity, resistance to endotoxin lethality was also studied. Animals made

tolerant by the method of Beeson (1) and those previously given a massive dose of endotoxin when they were young, were injected with several lethal doses of homologous endotoxin. Results given in Table II compare adult animals, which had been pretreated, with their normal litter mates at the same weight for lethal resistance to endotoxins; those that had previously received massive doses of endotoxin were all resistant to 4 mg/kg or 8 to 16 LD<sub>50</sub>, while all the normal litter mates died (A). Likewise, adult animals made tolerant by repeated daily injections of endotoxin were also completely resistant to 2 mg/kg of toxin or 4 to 8 LD<sub>50</sub> (B).

Resistance of pyrogenic tolerant animals to the lethal effect of endotoxin is shown by another approach given in Fig. 9. Here, both tolerant and control animals gave characteristic fever responses to 100 MPD-3/kg. After 4 hours, both groups were given an additional 250 µg/kg of the same endotoxin. The animals in the control group were immediately shocked and over 50 per cent died while the tolerant group gave a normal fever response and all survived. It should be emphasized that the febrile response in the tolerant group is that which would be given by 100 to 200 MPD-3/kg. The 250 µg/kg dose represents 25,000 MPD-3/kg, and it is apparent, therefore, that these animals were able to inactivate most of the injected toxin. In these tolerant animals, the mechanism which initiated the normal pyrogenic response remained intact and unmodified as indicated by the typical biphasic fever response.

#### DISCUSSION

The results presented in this study suggest that immunological mechanisms play a role in pyrogenic tolerance and other biological activities of endotoxins.

Based on these findings and those of others, the following concept is presented for discussion: Endotoxins have two interdependent activities, a primary and a secondary toxicity. The latter is determined by the hypersensitive state of the host to some portion of the macromolecular endotoxin. There is evidence that these two activities are in different parts of the macromolecule. Endotoxin tolerance, therefore, can be induced in two ways: one, by inactivating the primary toxicity, and two, by desensitizing the animal to the sensitizing portion of the endotoxin. The sensitizing determinant represents a common configuration in most endotoxins and is present in the cell wall of many microorganisms. Hypersensitivity to this determinant develops in most "normal" animals from contact with intestinal flora, subclinical and clinical infections. The acquired hypersensitivity or secondary toxicity is manifested by an enhancement of the 3 hour portion of the fever curve, enhanced susceptibility to the lethal effects and increased skin reactivity to endotoxins. The induction of pyrogenic tolerance by the repeated injection of endotoxin suppresses the 3 hour portion of the febrile response which correlates with acquired lethal tolerance. This desensitization or tolerance develops concomitantly with the acquisition of circulating classical antibodies; these assist the normally functioning RES to destroy



the endotoxin before its reactive groups contact the hypersusceptible cells of the host. The principle of this concept is not unlike that proposed by Cooke *et al.* (19) where blocking antibodies prevent the antigen from reacting with the skin sensitizing antibodies. The results used to formulate this concept will be presented and discussed in the order given above.

The existence of at least two activities associated with a single endotoxin macromolecule is indicated by the cross-tolerance studies with COO8 endotoxin and its derivative, Lipid A. Animals made tolerant to Lipid A showed little tolerance to the parent lipopolysaccharide (Fig. 3 A). One might assume that Lipid A has a low content of the major sensitizing portion of the parent toxin and a relatively high concentration of the configuration responsible for primary toxicity. Thus, it is possible that the parent toxin COO8 has a sensitizing antigen of a different specificity than that present in Lipid A; perhaps a common antigenic determinant was destroyed during the cleavage. If this were true, Lipid A should not make animals tolerant to the parent COO8, but the parent COO8 should induce tolerance to Lipid A. The correlation is nearly complete but the parent toxin does not give the anticipated complete tolerance to Lipid A (Fig. 3 A and B). Perhaps the dissociation of the Lipid A from the lipopolysaccharide exposed new determinant groups ordinarily present but not exposed in the parent COO8 toxin. Nowotny (20) assumed that new configurations may be present in Lipid A that are not evident in the parent lipopolysaccharide; this is explained by the reorientation and modification of reactive groups resulting from acid hydrolysis used in the cleavage. Regardless of the interpretation, the absence of complete cross-tolerance between these related toxins makes it difficult to attribute toxicity to a single configuration within the toxin. There is also evidence that primary toxicity may exist independently of that associated with hypersensitivity (3, 21-23).

As shown in this investigation, the resistance of the young rabbit to pyrogenicity, lethality, and skin reactivity with the gradual increase in the susceptibility to all of these attributes of endotoxin activity suggests a role for hypersensitivity in the secondary toxicity of endotoxins. Others have also presented evidence which could implicate hypersensitivity in one or more biological activities of endotoxin (3, 17, 24).

If hypersensitivity is involved as postulated, then desensitization would correlate with the development of pyrogenic tolerance (17). The mechanism of desensitization in delayed hypersensitivity is not known but it has been suggested that repeated injections of the antigen eventually destroys all of the sensitized cells. Evidence against this mechanism as applied to pyrogenic tolerance is given in Fig. 9. Animals made tolerant to 100 MPD-3/kg when injected with an overwhelming dose of toxin gave a biphasic fever response indicating that susceptible cells were available for damage in the tolerant or desensitized animal.

In the concept outlined here, desensitization as applied to pyrogenic tolerance involves the induction of a classical immune mechanism. We have assumed that the determinant groups responsible for the pyrogenicity observed in the 3 hour portion of the febrile response are mostly identical or related in the sense of cross-reacting antigens which account for the apparent non-specific nature of pyrogenic tolerance. Specificity of pyrogenic tolerance demonstrated by controlled cross-tolerance

tests with endotoxins from *C. violaceum* (CV) and *E. coli* (COO8) (Fig. 2) suggests that endotoxins isolated from organisms belonging to more unrelated families may show greater antigenic differences. This observation is supported by the results of Mergenhausen and Jensen (25) concerning endotoxin tolerance in the mouse. Vaccination of mice with killed Gram-negative cocci of the genus *Veillonella* increased the resistance of the animals to the lethal effect of an endotoxin from the homologous organism but not to an endotoxin isolated from the unrelated *E. coli*.

These results were again confirmed by the suppression of the 3 hour portion of the febrile response in adult rabbits by their early exposure to massive doses of endotoxins. Thus, young animals given a LD<sub>50</sub> of *S. typhosa* (ST0901) endotoxin, when tested as adults, 2 months later, were tolerant to the homologous toxin but not to the heterologous CV toxin (Fig. 8). Again, these results suggest an immunological mechanism. Originally, we attributed this suppression to immunological paralysis. Further studies on lethality and skin reactivity revealed similarities to the tolerance developed in adults by the method of Beeson (1) (Tables I and II). We postulated that when rabbits were given a large parenteral antigenic stimulation their classical antibody-forming mechanism was readily activated by small quantities of antigen received later from natural exposure. Animals not stimulated by sufficient antigenic mass may develop primarily delayed hypersensitivity induced by small quantities of antigen available from natural sources. The significant anamnestic response shown by the development of complete tolerance within 2 days after a single injection of endotoxin in animals which had lost their tolerance is also suggestive of a classical immune response (Fig. 4).

Additional evidence to support this concept includes studies on the passive transfer of tolerance by serum or plasma (26-28). Freedman (27), who has done extensive work in this area, does not attribute these significant observations to the presence of antibodies; the results, however, are consistent with an immunological mechanism. The factors involved could be the same as the opsonins, unrelated to O antibodies, which assist the RES in the destruction of certain Gram-negative bacteria (29). Jenkin and Rowley reported that such opsonins are removed non-specifically from the serum by colloidal carbon. The removal may be more specific than suspected since it has been shown that carbon from the same source (Special Biological Ink C11 1431a, Gunther Wagner, Germany, contains small quantities of pyrogens (30). Benacerraf and Miescher (31) have also discussed non-specific opsonins which were thought to be active against some unsuspected cross-reacting antigen present in most endotoxins.

Certainly many investigators (32) agree, as originally shown by Morgan (33), that antibodies against the O specific polysaccharides play no role in this mechanism. Also one must agree that the RES plays an important part in destroying the endotoxin (1, 34-36); this is not unique for endotoxin and is equally applicable to many immune mechanisms where specific antibodies assist the normally functioning RES. Thus, the ability to break pyrogenic tolerance by blocking the RES with thorotrast does not preclude a specifically acquired immune mechanism. The recent results of Stuart and Cooper (37) question the exclusive role of the RES in the mechanism of pyrogenic tolerance as originally suggested (1). The results of Greisman *et al.* (28) support the concept under discussion. They proposed that the tolerant animal possesses a

dual mechanism which includes the RES and a humoral factor thought to prepare the endotoxins for phagocytosis.

It is possible that some of the inconsistencies reported on the nature of endotoxins and their activities (38) can be explained on the basis of the two activities discussed. In addition to the complexity of the toxins, the host presents several additional variables. As shown in this investigation and stressed by others (3, 17), the importance of the immunological state of the host and its contributions to the various biological activities of endotoxin cannot be overemphasized.

#### SUMMARY

Evidence is presented suggesting that the apparent non-specificity of pyrogenic tolerance observed with Gram-negative bacterial endotoxins is due to related antigenic determinants associated with the macromolecular toxins. This is based on results obtained in rabbits from pyrogenic cross-tolerance tests with selected endotoxins. In these tests, purified endotoxins from *Escherichia coli* (COO8) and *Chromobacterium violaceum* (CV) gave results one might expect with non-reciprocal cross-reacting antigens in classical immune systems. Additional evidence for an immune mechanism in tolerance is suggested by the highly significant anamnestic response observed.

Lipid A, a toxic derivative of the purified COO8 endotoxin, failed to induce pyrogenic tolerance against the parent toxin. These results are explained by assuming that endotoxins have two interdependent activities associated with different portions of the macromolecule; one is assumed to be responsible for the primary toxicity, and the other is involved in secondary toxicity. The latter is dependent on the hypersensitive state of the host. Additional evidence for the role of hypersensitivity in secondary toxicity is based on the observation that adult rabbits are highly sensitive to the pyrogenic, lethal, and skin-reacting activities of endotoxin in contrast to young animals which are more resistant to all of these attributes of toxicity.

In adults, the host responses to pyrogenicity, lethality, and skin reactivity could be partially inhibited by the early exposure of the animals to massive doses of endotoxin equivalent to a  $LD_{50}$ . The pyrogenic tolerance shown in these animals was specific indicating that the inhibition of the hypersusceptibility to endotoxin involved an immunological mechanism.

A mechanism of endotoxin tolerance is proposed and discussed based on the induction of specific antibodies capable of assisting the RES in the clearance and destruction of endotoxin.

It is suggested that the present inconsistencies relative to the chemical nature and biological activities of endotoxins might be explained on the basis of these two activities and the failure to recognize the importance of the immunological state of the host in which the toxins are tested.

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